SUMMARY

A study was made pertaining to the variations in sensitivity to inhibitors that may be encountered in industrial deterioration problems. The results indicate that at least three types of variations are involved: first, the variation in sensitivity of different strains, secondly, the variation in sensitivity of an individual strain, and finally, the variation produced by the particular environment in which the inhibitor is studied.

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Physiology of Toxin Production by *Clostridium botulinum* Types A and B

II. Effect of Carbohydrate Source on Growth, Autolysis, and Toxin Production

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The kinetics of botulinum toxin production is as yet a comparatively ill-defined process. Bonventre and Kempe (unpublished observations) have recently found that the appearance of toxin in the culture filtrates of *Clostridium botulinum* types A and B is a function not of cellular growth but of the degree of autolysis. This substantiates the report by Stone (1954), Boroff (1955), Kindler *et al.* (1955), and others that autolysis is a mechanism of toxin liberation by many of the sporeforming anaerobic bacilli. This investigation concerns itself with the effects of the exogenous carbohydrate source on the growth, autolytic and toxin synthesizing processes of *C. botulinum* types A and B.

MATERIALS AND METHODS

The organisms utilized in this study are designated as *C. botulinum* type A strain JTD-IV;[2] *C. botulinum* type B, strain B-201;[2] *C. botulinum* type A, strain 62-A (NCA) and *Clostridium parabotulinum* type A, strain 457-A.

A complete medium consisting of 2.0 per cent tryptic digest of casein[3] and 0.5 per cent autolyzed yeast extract was employed for the cultivation of the organisms. After sterilization, the carbohydrate was added in the form of a 20 per cent sterile solution to give a final concentration of 0.5 per cent. Growth was measured turbidimetrically with a Klett-Summerson photoelectric colorimeter (model 800-3)[4] at 600 mμ.

Toxin assay. Cell-free culture fluids were obtained by centrifugation or Seitz filtration at 4 C. Owing to the lability of the toxin, certain precautions were taken during manipulation of the samples to be assayed. Dilutions were made in cold, sterile 0.5 per cent gelatin M/15 phosphate buffer at pH 6.8. Toxicity was established by intraperitoneal inoculation of the diluted

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[4] Klett Manufacturing Company, New York, New York.

samples into white mice of 20 to 25 g, and toxin titers were then calculated as minimum lethal doses (MLD/ml) according to the method of Wadsworth (1947).

Results and Discussion

Effect of glucose concentration. Preliminary experiments (Bonventre, 1957) showed that the rate of autolysis of *C. botulinum* was affected by the concentration of glucose in the growth medium. Maximum toxin synthesis was obtained at concentrations of glucose as low as 0.1 per cent if the cultures were incubated long enough to allow complete autolysis. It was also found that a complete medium supported the growth of *C. botulinum* in the absence of the hexose.

C. botulinum strains JTD-IV and B-201 were grown in the complete medium containing 0.5 per cent glucose and also in the same medium to which no glucose was added. After adaptation to growth under these conditions, toxin production was determined by assaying the culture filtrates. From the data in table 1 it is seen that the absence of glucose had a profound effect on the synthesis of toxin by both strains. The absence of the carbohydrate resulted in an approximate 1000-fold decrease in the toxicity of the filtrates. Autolysis in the absence of glucose did not go to completion even on prolonged incubation at 35 C. This suggested that the toxin might not have been detected if it were present intracellulary. Sonic disintregation of the cells, however, resulted in a negligible increase in the toxicity. This indicated that glucose was required, at least in low concentrations, for the synthesis of toxin as well as for the synthesis of the autolytic enzyme system. With B-201, the less toxigenic strain of the organism, toxin synthesis in absence of glucose was negligible.

Growth and toxin synthesis in the presence of various carbohydrate sources. Growth and toxin synthesis by four strains of *C. botulinum* were followed in the complete medium containing a utilizable carbohydrate source. Strains JTD-IV, 62-A, 457-A, and B-201 were tested for their ability to ferment the carbohydrate sources added. This was determined in phenol-red broth supplemented with the test carbohydrate at a concentration of a 0.5 per cent. Large Pyrex test tubes containing 20 ml of medium were deoxygenated by boiling for 10 min, cooled, and inoculated with 1 ml of an 18-hr culture. Transfers were made every 24 hr for 3 days to adapt the organism to the carbohydrate and to dilute out any contamination by glucose originating from the initial inoculum.

After the fourth transfer, growth was measured turbidimetrically and samples were taken periodically for toxin assay. It was found that glucose and maltose supported toxin synthesis fully. Of the other carbohydrates used, only glycerol, pyruvate and ribose partially supported synthesis of toxin. The quantity of toxin produced in the presence of galactose, lactose, xylose,

TABLE 1

Toxin synthesis by Clostridium botulinum strains JTD-IV and B-201 in the presence and in the absence of glucose

Age of Culture (hr)	Toxin Titers (MLD/ml)			
	Culture filtrate		Culture extract	
	With glucose	No glucose	With glucose	No glucose
JTD-IV				
20	5×10^3	10^2	1×10^4	1×10^3
48	1×10^5	10^3	5×10^5	1×10^3
72	2×10^5	10^3	1×10^6	1×10^3
B-201				
20	5×10^2	Neg	—	—
48	1×10^3	Neg	—	—
72	1×10^4	Neg	—	—
96	5×10^4	Neg	1×10^5	10

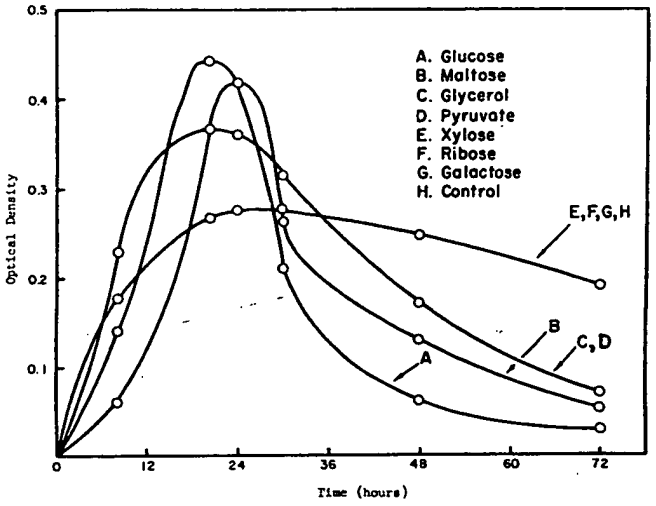

Figure 1. Effect of external carbohydrate source on growth of *Clostridium botulinum* strain JTD-IV.

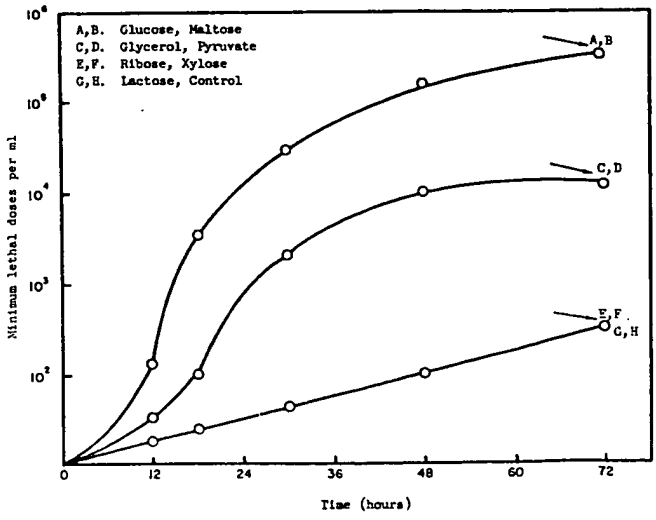

Figure 2. Effect of external carbohydrate source on toxin synthesis by *Clostridium botulinum* strain JTD-IV.

and inositol was no greater than in the control medium which contained no added carbohydrate.

Growth and toxin synthesis by strain JDT-IV, which may be taken as representative for all the strains tested, are presented graphically in figure 1 and 2, respectively. Although equal growth rates were observed during the exponential growth phase with all the carbohydrates, maximum growth was achieved only with glucose and maltose. The degree to which growth was repressed with the other carbon compounds when compared to glucose, however, was not sufficient to account for the extreme differences in toxin production. It can also be noted that autolysis of the culture did not go to completion unless glucose or maltose was included in the growth medium. Sonic disintregation of the intact cells, which remained after 72 hr of incubation in the media containing carbohydrates other than glucose and maltose, did not result in the increased toxicity of the supernatant fluid showing that the toxin was not present intracellularly.

SUMMARY

The data obtained from this survey indicate that glucose is essential for optimal toxin synthesis and that the requirement for this hexose cannot be replaced by the other energy sources tested.

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Physiology of Toxin Production by *Clostridium botulinum* Types A and B

III. Effect of pH and Temperature During Incubation on Growth, Autolysis, and Toxin Production

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The ever present hazard of botulinus intoxication has stimulated continuing research by various segments of the food industries. The fact that commercially prepared foods have been only rarely incriminated in botulinum outbreaks during the past three decades is testimony to the effectiveness of the research which has been carried out to determine the proper measures for avoiding such occurrences. The literature is voluminous regarding the upper and lower limits of pH and temperature which will support the growth of *Clostridium botulinum* in various natural food substrates and artificial media. However, these investigations are for the most part concerned with an all or none phenomenon, the organism multiplies and produces toxin or it does not. Ohye and Scott (1953, 1957) did quantitative growth determinations of *C. botulinum* types A, B, and E at various temperatures but did not attempt to correlate this with toxin production. Since autolysis has been shown to be correlated with the liberation of toxin by *C. botulinum* (Boroff, 1955; Kindler *et al.*, 1955; Bonventre and Kempe, 1959), it was considered that a quantitative study concerning the effects of pH and temperature incubation on the growth, autolysis, and toxin production by *C. botulinum* would be valuable from both a practical and theoretical point of view.

MATERIALS AND METHODS

C. botulinum type A strain JTD-IV[2] was used in this investigation. Growth was measured turbidimetrically, and toxin assayed as described previously by Bonventre and Kempe (1959).

pH of medium. The complete medium described by Bonventre and Kempe (1959) for the cultivation of all type A strains of *C. botulinum* was freshly prepared and

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